REMARKS

Applicant submits this Communication to the Examiner in order to provide further evidence of the impropriety of the rejections for obviousness on pages 10-18 of the Office Action mailed on March 12, 2009, in response to which an Amendment was timely filed on June 3, 2009. The application is not amended herein.

Rejections of the Claims for Obviousness under 35 U.S.C. §103(a)

A. Hermsmeyer, WO 98/37897; in view of Beaumont, Clin. Exp. Immunol., 24:455-463 (1976) (claims 1-3, 7, 9, 10, and 13-16)

B. Hermsmeyer, WO 98/37897; in view of Burghardt, Biology of Reproduction, 36:741-51 (1987); and Barkheim, Molecular Pharmacology, 54:105-112 (1998) (claims 1-3, 5-7, 9, 10, and 13-16)

C. Hermsmeyer, WO 98/37897; in view of Burghardt, Biology of Reproduction, 36:741-51 (1987); Barkheim, Molecular Pharmacology, 54:105-112 (1998); and Shaak, U.S. Patent No. 6,228,852 (claims 11 and 12)

D. Hermsmeyer, WO 98/37897; in view of Burghardt, Biology of Reproduction, 36:741-51 (1987); Barkheim, Molecular Pharmacology, 54:105-112 (1998); and Meyers, J. Med. Chem., 44:4230-4251 (2001) (claim 8)

In the Office Action of March 12, 2009, the Examiner rejected the claims, as indicated above, as being obvious in view of the disclosure of Hermsmeyer, in view of either Beaumont or Burghardt, and, in the case of Burghardt, in view of additional references Barkheim plus or minus either Shaak or Meyers.

Regarding the rejection of claims 1-3, 7, 9, 10, and 13-16 as being obvious in view of the combined disclosures of Hermsmeyer and Beaumont, the Examiner stated on page 10 of the Office Action that "Beaumont et al. teaches estriol and progesterone as steroids (Table 3)." and that "Beaumont et al. teaches the equivalence of estriol and progesterone as steroids."

Regarding the rejection of claims 1-3, and 7-16 as being obvious in view of the combined disclosures of Hermsmeyer, Burghardt, and Barkheim, plus or minus either Shaak or Meyers, the Examiner stated that "Burghardt et al. teaches progesterone, estradiol, 3βAdiol as estrogen receptor binding ligands (Table 2)." See the Office Action, pages 11, 13, and 14.

In the Amendment filed on June 3, 2009, in the bridging paragraph of pages 14-15 and in the first full paragraph of page 15, Applicant addressed the Examiner's contention that Beaumont discloses the equivalence of estriol and progesterone as steroids. In the last full paragraph of page 16 through the end of the first full full paragraph of page 17 of the Amendment, Applicant addressed the Examiner's contention that Burghardt discloses that progesterone is an estrogen receptor binding ligand.

In the present Communication, Applicant supplies proof, in the way of a scientific article, that progesterone and estrogens, such as estriol, are not equivalent steroids and that progesterone is not an estrogen receptor binding ligand.

Submitted herewith is Kuiper et al, "Interaction of Estrogenic Chemicals and Phytoestrogens with Estrogen Receptor β," Endocrinology, 139(10):4252-4263 (1998). Table 1 on page 4256 shows the relative binding affinity (RBA) for estrogen receptor α (ERα) and for estrogen receptor β (ERβ) of a list of compounds relative to the binding affinity of 17β estradiol for ERα and ERβ.

In Table 1, the binding affinity of 17β estradiol for ERα and ERβ is arbitrarily set at 100. A number of other estrogens, and estrogenic compounds are listed and show some degree of binding to ERα and ERβ.

The Examiner's attention is directed to the RBA for progesterone and testosterone shown in Table 1. Pertaining to either ERα or ERβ, neither progesterone nor testosterone demonstrated measurable binding to either estrogen receptor.

The Examiner's attention is further directed to the RBA for epiestriol (17β-epiestriol) shown in Table 1. The RBA (relative binding affinity) of epiestriol for ERα is 29 and for ERβ is 80, both of which are less than but comparable to the binding affinity of 17β-estradiol for these estrogen receptors.

Applicant submits that it is clear from the data of Table 1 of Kuiper that the statement of the Examiner based on the disclosure of Beaumont that estriol and progesterone are equivalent steroids is erroneous. Applicant further submits that it is clear from the data of Table 1 of Kuiper that the statement of the Examiner based on the disclosure of Burghardt that progesterone is an estrogen binding receptor ligand is also erroneous. Rather, Kuiper discloses that progesterone, like testosterone, does not bind appreciably to either estrogen receptor α or estrogen receptor β.

In view of the above, and in view of the arguments presented in the Amendment of June 3, 2009, Applicant respectfully requests the Examiner to reconsider and to withdraw the rejections of the claims, as indicated above, for obviousness based on the disclosures of Hermsmeyer in view of in view of Beaumont, or Hermsmeyer in view of Burghardt and Barkheim with or without additional references Shaak and Meyers.

E. Hermsmeyer, U.S. Patent No. 6,056,972; in view of Weihua, PNAS, 99:13589-13584 (2002)

In the Amendment filed June 3, 2009, Applicant inadvertently omitted the response to the rejection of claims 4-6 and 9-10 for obviousness in view of the combined disclosures of Hermsmeyer, U.S. Patent No. 6,056,972; and Weihua, PNAS, 99:13589-13584 (2002). Applicant addresses that basis of rejection herein and traverses the rejection of these claims on this ground.

The disclosure of Hermsmeyer U.S. Patent No. 6,056,972 is essentially the same as that of Hermsmeyer, WO 98/37897, as indicated by the Examiner in the present Office Action on page 16 where the Examiner states that "Hermsmeyer et al. teachings discussed as above." As indicated in the Amendment of June 3, 2009, Hermsmeyer does not disclose or suggest that a compound that is a selective ER-beta agonist would have any effect whatsoever on reducing vascular hyperreactivity. It is submitted, therefore, that the disclosure of Hermsmeyer has no relevance to the present invention or to the issue of patentability of the present invention.

Weihua is cited for its disclosure that androstane is a ERβ receptor agonist.

The combination of these references does not disclose or suggest the present invention and, therefore, the Examiner is respectfully requested to withdraw the rejection of claims 4-6 and 9-10 on this ground.

F. Hermsmeyer, U.S. Patent No. 6,056,972; in view of Barkheim, Molecular Pharmacology, 54:105-112 (1998)

In the Amendment filed June 3, 2009, Applicant inadvertently omitted the response to the rejection of claim 7 for obviousness in view of the combined disclosures of Hermsmeyer, U.S. Patent No. 6,056,972; and Barkheim, Molecular Pharmacology, 54:105-112 (1998). Applicant addresses that basis of rejection herein and traverses the rejection of this claim on this ground.

Hermsmeyer discloses that administration of progesterone is useful in treating coronary arterial vasospasm. As indicated by the Examiner, Hermsmeyer does not disclose an ER-beta agonist compound to reduce vascular reactivity. As indicated in the Amendment of June 3, 2009, Hermsmeyer does not disclose or suggest that a compound that is a selective ER-beta agonist would have any effect whatsoever on reducing vascular hyperreactivity. It is submitted, therefore, that the disclosure of Hermsmeyer has no relevance to the present invention or to the issue of patentability of the present invention.

Barkheim discloses the existence of two different estrogen receptors, the estrogen alpha and the estrogen beta receptor, and further discloses that estradiol has selective alpha agonist potency and that epiestriol has selective beta agonist potency.

As argued in the Amendment of June 3, 2009, the relevance of Barkheim to the present application is limited to its disclosure that epiestriol is a estrogen beta-receptor agonist. However, the prior art, does not suggest or disclose that an estrogen beta-receptor agonist can be used successfully in a method as called for in the present claims. Accordingly, the Examiner is respectfully requested to reconsider and to withdraw the rejection of claim 7 on this ground.

ADDITIONAL REMARKS

In the Conclusion section of the Amendment of June 3, 2009, Applicant stated that a Request for Continued Examination was being submitted. That statement was in error because the Office Action of March 12, 2009 was not a final rejection. For clarification, no Request for Continued Examination was submitted with the Amendment of June 3, 2009.

CONCLUSION

Applicant submits that the claims, as amended in the Amendment of June 3, 2009, are in condition for allowance and requests an early notice to that effect. Applicant submits a Request for Continued Examination, with applicable fees, with this Amendment.

Respectfully submitted,

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Attachment: Kuiper et al, Endocrinology, 139(10):4252-4263 (1998).

CERTIFICATE OF TRANSMISSION/MAILING

I hereby certify that this correspondence is being facsimile transmitted to the Patent and Trademark Office at (571) 273-8300 or deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Commissioner for Patents, Box 1450, Alexandria, VA 22313-1450 on July 7, 2009.

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Howard M. Eisenberg

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Interaction of Estrogenic Chemicals and Phytoestrogens with Estrogen Receptor β

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ABSTRACT

The rat, mouse and human estrogen receptor (ER) exists as two subtypes, ERα and ERβ, which differ in the C-terminal ligand-binding domain and in the N-terminal transactivation domain. In this study, we investigated the estrogenic activity of environmental chemicals and phytoestrogens in competition binding assays with ERα or ERβ protein, and in a transient gene expression assay using cells in which an acute estrogenic response is created by cotransfecting cultures with recombinant human ERα or ERβ complementary DNA (cDNA) in the presence of an estrogen-dependent reporter plasmid.

Saturation ligand-binding analysis of human ERα and ERβ protein revealed a single binding component for [^{3}H]-17β-estradiol (E_2) with high affinity (dissociation constant (K_d) = 0.05 - 0.1 nM). All environmental estrogenic chemicals [polychlorinated hydroxybiphenyls, dichlorodiphenyltrichloroethane (DDT) and derivatives, alkylphenols, bisphenol A, methoxychlor and chlordecone] compete with E_2 for binding to both ER subtypes with a similar preference and degree. In most instances the relative binding affinities (RBA) are at least 1000-fold lower than that of E_2. Some phytoestrogens such as coumestrol, genistein, apigenin, naringenin, and kaempferol compete stronger with E_2 for binding to ERβ than to ERα. Estrogenic chemicals, as for instance nonylphenol, bisphenol A, o, p'-DDT and 2',4',6'-trichloro-4-biphenylol stimulate the transcriptional activity of ERα and ERβ at concentrations of 100-1000 nM. Phytoestrogens, including genistein, coumestrol and zearalenone stimulate the transcriptional activity of both ER subtypes at concentrations of 1–10 nM. The ranking of the estrogenic potency of phytoestrogens for both ER subtypes in the transactivation assay is different; that is, $E_2 \gg$ zearalenone = coumestrol > genistein > daidzein > apigenin = phloretin > biochanin A = kaempferol = naringenin > formononetin = ipriflavone = quercetin = chrysin for ERα and $E_2 \gg$ genistein = coumestrol > zearalenone > daidzein > biochanin A = apigenin = kaempferol = naringenin > phloretin = quercetin = ipriflavone = formononetin = chrysin for ERβ. Antiestrogenic activity of the phytoestrogens could not be detected, except for zearalenone which is a full agonist for ERα and a mixed agonist-antagonist for ERβ. In summary, while the estrogenic potency of industrial-derived estrogenic chemicals is very limited, the estrogenic potency of phytoestrogens is significant, especially for ERβ, and they may trigger many of the biological responses that are evoked by the physiological estrogens. (*Endocrinology* 139: 4252–4263, 1998)

THE STEROID hormone estrogen influences the growth, differentiation, and functioning of many target tissues. These include tissues of the female and male reproductive systems such as mammary gland, uterus, vagina, ovary, testes, epididymis, and prostate (1). Estrogens also play an important role in bone maintenance, in the central nervous system and in the cardiovascular system where estrogens have certain cardioprotective effects (1–4). Estrogens diffuse in and out of cells but are retained with high affinity and specificity in target cells by an intranuclear binding protein, termed the estrogen receptor (ER). Once bound by estrogens, the ER undergoes a conformational change allowing the receptor to interact with chromatin and to modulate transcription of target genes (5–7). We have cloned a novel ER cDNA from rat prostate (8), named ERβ, different from the previously cloned ER cDNA (consequently renamed ERα). Rat ERβ cDNA encodes a protein of 485 amino acid residues with a calculated molecular weight of 54200. Rat ERβ protein is highly homologous to rat ERα protein, particularly in the DNA binding domain (95% amino acid identity) and in the C-terminal ligand binding domain (55% homology). In addition, recently a variant rat ERβ cDNA was cloned that has an in-frame insertion of 54 nucleotides that results in the predicted insertion of 18 amino acids within the ligand-binding domain (9, 10). Mouse (11, 12) and human homologs (13, 14) of rat ERβ have been cloned, and similar homologies in the various domains of the subtypes were found. Expression of ERβ was investigated by Northern blotting, RT-PCR, and *in situ* hybridization; prominent expression was found in prostate, ovary, epididymis, testis, bladder, uterus, lung, thymus, colon, small intestine, vessel wall, pituitary, hypothalamus, cerebellum, and brain cortex (4, 10–16a). Saturation ligand binding experiments revealed high affinity and specific binding of 17β-estradiol (E_2) by ERβ protein, and ERβ is able to stimulate transcription of an estrogen response

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element containing reporter gene in an E_2-dependent manner (10–13, 15). More extensive studies showed that some synthetic estrogens and naturally occurring steroidal ligands have different relative affinities for ERα *vs.* ERβ, although most ligands (including various antiestrogens) bind with very similar affinity to both ER subtypes (15).

There is increasing concern over the putative effects of various chemicals released into the environment on the reproduction of humans and other species. Threats to the reproductive capabilities of birds, fish, and reptiles have become evident and similar effects in humans have been proposed (17–21). In the past 50 yr, the incidence of testicular cancer and developmental male reproductive tract abnormalities appear to have increased in some developed countries (19). Several reports have also provided evidence for a decline in semen quality and/or sperm count over the same period, although this change may not be universal (19 and references therein). Male offspring born to mothers who were given diethylstilbestrol (DES), a very potent synthetic estrogen, to prevent miscarriages have an increased incidence of undescended testes, urogenital tract abnormalities, and reduced semen quality compared with those from mothers who did not take DES (22 and references therein). In mice injected with DES between days 9 and 16 of gestation, there is an increased risk of intraabdominal testes, sterility, and abnormalities in the urogenital tract of the offspring (22 and references therein). The similarities between the observations made in DES offspring and the abnormalities being observed in the general population have led to the hypothesis that one potential cause of the rise in male reproductive tract abnormalities might be inappropriate exposure to estrogens or suspected environmental estrogenic chemicals (from pesticides, components of plastics, hand creams, etc.) especially during fetal and/or neonatal life (17–21). Examples of suspected environmental estrogenic chemicals include OH-PCBs (polychlorinated hydroxybiphenyls), DDT and derivatives, certain insecticides and herbicides as Kepone and methoxychlor, certain plastic components as bisphenol A, and some components of detergents and their biodegradation products as, for instance, alkylphenols (17–21, 23–29). All these compounds bind weakly to the ERα protein extracted from rat uterus or human breast tumor cells or with recombinant ERα protein (23–29). No data are yet available on the potential interaction of estrogenic chemicals with ERβ, and interactions of xenoestrogens with this subtype may be related to some recent observations. In the rat and mouse prostate, ERβ messenger RNA (mRNA) is highly expressed in the secretory epithelial cells (8, 30), and it has been shown that fetal or neonatal exposure to E_2/DES or estrogenic chemicals causes not only permanent changes in the size of the prostate but also in the expression level of certain genes (30–32). In the fetal rat testis, ERβ is expressed in Sertoli cells and gonocytes (33), and maternal exposure to DES or 4-octylphenol alters the expression of steroidogenic factor I (SF-1) in Sertoli cells of the fetal rat testis (34). In the human midgestational fetus, high amounts of ERβ mRNA are present in the testes, but the cellular localization is unknown (35).

Human diet contains several plant-derived, nonsteroidal weakly estrogenic compounds (1). They are either produced by plants themselves (phytoestrogens), or by fungi that infect plants (mycoestrogens). Chemically, the phytoestrogens can be divided into three main classes: flavonoids (flavones, isoflavones, flavanones and chalcones) such as genistein, naringenin, and kaempferol; coumestans (such as coumestrol); and lignans (such as enterodiol and enterolactone). Mycoestrogens are mainly zearalenone (resorcylic acid lactone) or derivatives thereof, which have been associated with estrogenizing syndromes in cattle fed with mold-infected grain (1). Phytoestrogens and mycoestrogens act as weak mitogens for breast tumor cells *in vitro*, compete with 17β-estradiol for binding to ERα protein, and induce activity of estrogen-responsive reporter gene constructs in the presence of ERα protein (36–38). Intake of phytoestrogens is significantly higher in countries where the incidence of breast and prostate cancers is low, suggesting that they may act as chemopreventive agents (39). The chemopreventive effect of dietary soy, which is rich in phytoestrogens, has been demonstrated on the development of chemically or irradiation-induced mammary tumors in mice (39 and references therein), and as a delayed development of dysplastic changes in the prostate of neonatally estrogenized mice (40). The expression of ERβ in rat, mouse, and human prostate might be of importance in this regard. Phytoestrogens are believed to exert their chemopreventive action by interacting with estrogen receptors, although alternative mechanisms, most notably inhibition of protein tyrosine kinase activity, have been proposed (39, 41).

In the present study, we have evaluated the estrogenic activity of suspected environmental estrogens and phytoestrogens in competition binding assays with ERα or ERβ protein, and in a transient gene expression assay using cells in which an acute estrogenic response is created by cotransfecting cultures with recombinant human ERα or ERβ cDNA in the presence of an estrogen-dependent reporter plasmid.

Materials and Methods

Materials

The steroids 17β-estradiol, 17α-estradiol (1, 3, 5(10)-estratriene-3,17α-diol), 16-keto-17β-estradiol (1, 3, 5(10)-estratriene-3,17β-diol-16-one), 17-epiestriol (1, 3, 5(10)-estratriene-3,16α,17α-triol), 16α-bromoestradiol (1, 3, 5(10)-estratriene-16α-bromo-3,17β-diol), 2-OH-estrone (1, 3, 5(10)-estratriene-2,3-diol-17-one), progesterone, 5-androstenediol (5-androstene-3β, 17β-diol) and testosterone were obtained from Steraloids Inc. (Wilton, NH).

The synthetic estrogen diethylstilbestrol (4, 4'-(1, 2-diethyl-1, 2-ethene-diyl)-bisphenol) was obtained from Steraloids. The antiestrogens tamoxifen (1-*p*-β-dimethylamino-ethoxy-phenyl-*trans* -1,2-diphenyl-1-butene), 4-OH-tamoxifen (1-(*p*-dimethylaminoethoxy-phenyl)1-(4-hydroxyphenyl)-2-phenyl-1-butene), raloxifene (6-hydroxy-3-[4-[2-(1-piperidinyl)ethoxy]phenoxy]-2-(4-hydroxy phenyl)-benzothiophene) and ICI-164384 (*N*-*n*- butyl-11-(3, 17β-dihydroxyestra-1, 3, 5(10)trien-7α-yl)-*N*-methyl-undecanamide) were obtained from Sigma Chemical Co. (St. Louis, MO) or synthesized by KaroBio AB. The steroidal antiestrogen ICI-182780 was kindly supplied by Zeneca Pharmaceuticals (Cheshire, UK).

The flavonoids genistein (4', 5, 7-trihydroxyisoflavone), daidzein (4', 7-dihydroxyisoflavone), formononetin (7-hydroxy-4'-methoxyisoflavone), biochanin A (5, 7-dihydroxy-4'-methoxyisoflavone), apigenin (4', 5, 7-tri-hydroxyflavone), chrysin (5, 7-dihydroxyflavone), kaempferol (3, 4', 5, 7-tetrahydroxyflavone), quercetin (3, 3', 4', 5, 7-pentahydroxyflavone), naringenin (4', 5, 7-trihydroxyflavanone), phloretin (2', 4, 6'-trihydroxy-3-(p-hydroxyphenyl)-propiophenone), ipriflavone (7-isopropoxyisoflavone), and the nonhydroxylated compound flavone (2-phenyl-1, 4-benzopyrone) were obtained from Sigma or Roth Chemi-

calien (Karlsruhe, Germany). The phytoestrogen coumestrol (2-(2, 4-dihydroxyphenyl)-6-hydroxy-3-benzofurancarboxylic acid lactone) was obtained from Eastman Kodak (Rochester, NY) and zearalenone (6-[10-hydroxy-6-oxo-trans-1-undecenyl)-2,4-dihydroxybenzoic acid lactone) from Sigma.

The insecticide DDT and metabolites 2,4'-DDT/o, p'-DDT (1-chloro-2-(2, 2, 2-trichloro-1-(4-chlorophenyl)ethyl)benzene), 4,4'-DDT/p, p'-DDT (1, 1'-(2, 2, 2-tri-chloroethylidene)bis(4-chlorobenzene)), 2,4'-DDE/o, p'-DDE (2(2-chloro-phenyl)-2-(4-chlorophenyl)-1,1-dichloroethylene), 4,4'-DDE/p, p'-DDE (1, 1'-(dichloroethenylidene)-bis(4-chlorobenzene)), 2,4'-TDE/o, p'-TDE (1-chloro-2-(2, 2-dichloro-1-(4-chlorophenyl)ethyl)-benzene), 4,4'-TDE/p, p'-TDE (1, 1'-(2, 2-dichloroctylidene)-bis(4-chlorobenzene)), chlordecone (Kepone) (decachloro-octahydro-1,3,4-metheno-2*H*-cyclobuta(*cd*)pentalene), endosulfan (1, 4, 5, 6, 7, 7-hexachloro-5-norbornene-2, 3-dimethanol cyclic sulfite) and methoxychlor (1, 1, 1-trichloro-2, 2-bis(p-methoxyphenyl)ethane) were obtained from CIIT (Chemical Industry Institute of Toxicology, Research Triangle Park, NC). The plastic component bisphenol A (2, 2-bis(4-hydroxy-phenyl)propane) and the alkylphenolic compounds 4-*tert*-octylphenol, 4-octylphenol, 4-*tert*-amyl-phenol, 4-*tert*-butylphenol and nonylphenol were obtained from Aldrich (Tyresö, Sweden).

The hydroxylated polychlorinated biphenyl (OH-PCB) congeners OH-PCB-A (2, 2', 3', 4', 5'-pentachloro-4-biphenylol), OH-PCB-B (2, 2', 3', 4', 6'-pentachloro-4-biphenylol), OH-PCB-C (2, 2', 3', 5', 6'-pentachloro-4-biphenylol), OH-PCB-D (2, 2', 4', 6'-tetrachloro-4-biphenylol), OH-PCB-E (2', 3, 3', 4', 5'-pentachloro-4-biphenylol), OH-PCB-F (2', 3, 3', 4', 6'-pentachloro-4-biphenylol), OH-PCB-G (2', 3, 3', 5', 6'-pentachloro-4-biphenylol), OH-PCB-H (2', 3, 4', 6'-tetrachloro-4-biphenylol), OH-PCB-K (2', 4', 6'-trichloro-4-biphenylol), OH-PCB-L (2', 3', 4', 5'-tetrachloro-4-biphenylol), OH-PCB1 (2, 3, 3', 4', 5-pentachloro-4-biphenylol), OH-PCB2 (2, 2', 3, 4', 5, 5'-hexachloro-4-biphenylol), OH-PCB3 (2, 2', 3', 4, 4', 5, 5'-heptachloro-3-biphenylol), OH-PCB4 (2', 3, 3', 4', 5-pentachloro-4-biphenylol), OH-PCB5 (2, 2', 3, 3', 4', 5-hexachloro-4-biphenylol), OH-PCB6 (2, 2', 3, 3', 4', 5, 5'-heptachloro-4-biphenylol) and OH-PCB7 (2, 2', 3, 4', 5, 5', 6-heptachloro-4-biphe-nylol) were synthesized via Cadogan coupling as described (42, 43). The purity was greater than 98% as determined by gas-liquid chromatography. The nonchlorinated compounds 4,4'-biphenol and 4-biphenylol were obtained from Aldrich. The structural formula and chemical properties of the compounds used can be found in the Merck Index or elsewhere (1, 37, 41–43).

Expression and generation of ERα and ERβ protein extracts

A 1.5-kb DNA fragment encoding the human homolog of rat ERβ protein (485 amino acid residues) was excised with *Sac*II/SpeI from pGEM-T/hERβ (14) and isolated from agarose gel. The fragment was ligated to a *Bam*HI/*Sac*II adapter, recut with *Bam*HI/SpeI and ligated into the *Bam*HI/*Xba*I sites of the baculovirus donor vector pFastBac 1 (Life Technologies, Gaithersburg, MD). Recombinant baculovirus was generated using the BAC-TO-BAC expression system (Life Technologies) in accordance with manufacturer's instructions.

The human ERα coding sequence was derived from the mammalian expression vector pMT-hERα. The plasmid was linearized with *Sac*I, and a *Bam*HI linker was ligated after T4 DNA-polymerase treatment. The 1.9-kb fragment encoding hERα was excised with *Bam*HI and cloned into the baculovirus transfer vector pVL941 (kindly provided by Dr. M. D. Summers, Texas A&M University, College Station, TX). The recombinant transfer vector pVL941/hERα was cotransfected together with wild-type AcNPV DNA into Sf9 cells and polyhedrin negative plaques were isolated after several rounds of plaque purification. The recombinant baculoviruses were amplified and used to infect Sf9 cells. Infected cells were harvested 48 h post infection. A nuclear fraction was obtained as described (44), the resulting nuclei were extracted with buffer (17 mM K_2HPO_4, 3 mM KH_2PO_4, 1 mM $MgCl_2$, 0.5 mM EDTA, 6 mM monothioglycerol, 400 mM KCl, 8.7% glycerol; pH = 7.6) and the concentration of ER protein in the extract was measured as specific ^{3}H-17β-estradiol binding with the solubilized receptor based assay (see below). The ERα extract contained 400 pmol receptor/ml and the ERβ extract contained 800 pmol receptor/ml. The extracts were aliquoted and stored at −80 C.

Nonseparation solid-phase ligand binding competition experiments

These experiments were performed as described (45). In brief, the nuclear extracts were diluted (ERα extract 50-fold and ERβ extract 90-fold) in coating buffer (17 mM K_2HPO_4, 3 mM KH_2PO_4, 40 mM KCl, 6 mM monothioglycerol, pH 7.6). The diluted extracts (200 μl/well) were added to Scintistrip wells (Wallac Oy, Turku, Finland) and incubated for 18 h at ambient temperature.

Following noncovalent adhesion of receptor proteins the wells were washed twice with buffer A (17 mM K_2HPO_4, 3 mM KH_2PO_4, 140 mM KCl, 6 mM monothioglycerol, pH 7.6). Serial dilutions of the compounds to be tested were made in DMSO to concentrations 50-fold higher than the desired final concentrations. The DMSO solutions were diluted 50-fold in buffer A containing 3 nM ^{3}H-17β-estradiol [NEN-Life Science Products, Boston, MA; specific activity (S.A.) = 85 Ci/mmol]. The binding experiments were initiated by adding the incubation mixtures (175 μl) to the washed wells. Incubation was for 18 h at ambient temperature. The Scintistrip plates were counted in a MicroBeta counter fitted with six detectors (Wallac Oy, Turku, Finland). The data were evaluated by a nonlinear four-parameter logistic model (46) to estimate the IC_{50} value (the concentration of competitor at half-maximal specific binding). Relative binding affinity (RBA) of each competitor was calculated as the ratio of concentrations of E_2 and competitor required to reduce the specific radioligand binding by 50%, and the RBA value for E_2 was arbitrarily set at 100.

Ligand binding experiments with solubilized receptor using gel filtration for separation of bound and free radioligand

These experiments were performed, with minor modifications, as described previously (47). In brief: insect cell extracts were diluted in buffer B (20 mM HEPES, pH 7.5; 150 mM KCl, 1 mM EDTA, 6 mM monothioglycerol, 8.7% [vol/vol) glycerol) to a final ER concentration of 0.3–0.4 nM. Serial dilutions of the compounds to be tested were made in DMSO to concentrations 50-fold higher than the desired final concentrations. The DMSO solutions were diluted 50-fold with buffer B and ^{3}H-17β-estradiol (NEN-Life Science Products; S.A. = 85 Ci/mmol) was added to a final concentration of 3 nM. Unprogrammed rabbit reticulocyte lysate (Promega, Madison, WI; 1 μl/200 μl) was added to increase the protein concentration. Incubation was for 18–20 h at 6 C. Bound and free radioligand were separated on Sephadex G-25 columns as described (46), and the radioactivity in the eluate was measured after addition of 4 ml Wallac Supermix scintillation cocktail in a Wallac Rackbeta 1217 counter (Wallac Oy, Turku, Finland). The IC50 and RBA values were calculated as described above.

For saturation ligand binding analysis, the insect cell extracts were diluted to a final ER concentration of about 0.1 nM, and incubated for 18 h at 4 C with a range of ^{3}H-17β-estradiol (S.A. = 130 Ci/mmol) concentrations in the presence or absence of a 300-fold excess of unlabeled E_2. The dissociation constant (*Kd*) was calculated as the free concentration of radioligand at half-maximal specific binding by fitting data to the Hill equation (48) and by linear Scatchard transformation (49).

Transient gene expression assay in 293 human embryonal kidney cells

The estrogen-responsive reporter gene construct (3×ERE-TATA-LUC) which contains three copies of a consensus estrogen response element (ERE) containing oligonucleotide and a TATA box in front of the luciferase cDNA, is described in more detail elsewhere (van der Burg *et al.*, in preparation). The human ERβ expression plasmid pSG5-hERβ contains a 1.5 kb human ERβ cDNA, encoding the 485 amino acid residue human ERβ protein as described (14). The human ERα expression plasmid pSG5-HEGO (kindly provided by Dr. P. Chambon, IGBMC, Strasbourg, France) was used. Human 293 embryonal kidney cells were obtained from the ATCC (American Type Culture Collection, Rockville, MD), and cultured in a 1:1 mixture of DMEM and Ham's F12 medium (DF) supple-mented with 7.5% FCS. The cells were trypsinized and suspended in phenol red free DF medium containing 30 nM selenite, 10 μg/ml transferin and 0.2% BSA, supplemented with 5% charcoal stripped FCS. They were plated in 24 well tissue culture plates and 24 h later the cultures were transfected by the calcium phosphate precipita-

tion method (50) with 1 μg 3×ERE-TATA-LUC, 0.2 μg SV2-LacZ (51) internal control plasmid and 0.1 μg of the respective ER expression plasmid. After 16 h the medium was changed and the compounds to be tested (dissolved in ethanol) were added directly to the medium at a 1:1000 dilution. After 24 h, the cells were scraped in lysis solution (1% (vol/vol) Triton X-100, 25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA and 1 mM DTT). The luciferase activity of the cell lysates was measured with the Luclite luciferase reporter gene assay system (Packard Instruments, Meriden, CT) according to manufacturer's instructions, and the β-galactosidase activity was measured to correct for variations in transfection efficiencies (51).

Results

Expression and saturation ligand binding analysis of ER protein

Various steroid receptors including human ERα protein, have been expressed in large quantities in the baculovirus-Sf9 insect cell system and reported to be biologically active and structurally indistinguishable from the authentic receptor proteins (52). Furthermore, it has been demonstrated that posttranslational processing of proteins produced in Sf9 insect cells closely parallels these events in mammalian cells (53). It was therefore decided to use human ERα and ERβ protein expressed in insect cells for the ligand binding experiments.

In Fig. 1, the result of a saturation ligand binding experiment with [^{3}H]-17β-estradiol in the solubilized receptor ligand-binding system (see *Materials and Methods*) is shown. At the receptor concentrations employed (0.05–0.1 nM) the K_d values calculated from the saturation curves were 0.05 nM for ERα and 0.07 nM for ERβ protein. Linear transformation of saturation data (Scatchard plots in Fig. 1) revealed a single population of binding sites for 17β-estradiol with a K_d of 0.05 nM for the ERα protein and 0.09 nM for ERβ protein. In a previous report (15) we found a 4-fold higher affinity for ERα compared with ERβ, however, in that study 16α-[^{125}I]-iodo-17β-estradiol was used as ligand instead of [^{3}H]-17β-estradiol.

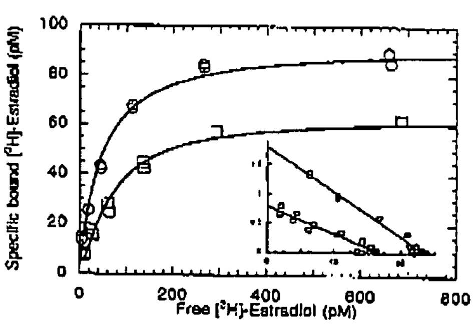

FIG. 1. Binding of ^{3}H-17β-estradiol to recombinant ERα and ERβ protein (solubilized receptor assay) in the presence or absence of a 300-fold excess of E_2 for 18 h at 6 C. Unbound radioligand was removed as described (solubilized receptor assay), and specific bound radioligand (ERα = O; ERβ = □) was calculated by subtracting nonspecific bound counts from total bound counts. *Inset*, Scatchard plot analysis of specific binding giving a K_d of 0.05 nM for ERα protein and a K_d of 0.09 nM for ERβ protein.

Ligand binding specificity of ERα and ERβ protein

Measurements of the equilibrium binding of the radioligand in the presence of different concentrations of unlabeled competitors provide readily interpretable information about the affinities of the latter. To group a large number of suspected endocrine disruptors and phytoestrogens into those which show significant affinity for both ER subtypes and those which do not bind at all, we used a previously developed solid-phase binding system as a screening assay (45). In the solid-phase binding assay recombinantly produced human ERα and ERβ proteins in insect cell extracts are attached to the wells of scintillating microtitration plates. The signal detection is based on the fact that ^{3}H emits low energy electrons that have a very short range in solution and therefore only radioligand bound to receptors triggers a scintillation process.

Overall ERα and ERβ show the relative binding affinities (Table 1) for the steroidal ligands and antiestrogens characteristic for an ER protein (1, 5, 15). The estradiol binding is stereospecific and the most potent synthetic estrogen DES binds with equal relative affinity to both ER proteins. The measured 7-fold greater affinity of 16α-bromo-17β-estradiol for ERα is in line with the measured 4-fold higher K_d (= lower affinity) of ERβ compared with ERα for the radioligand 16α-iodo-17β-estradiol (15). The selective estrogen receptor modulator (SERM) raloxifene and various E_2 metabolites (17-epiestriol and 16-keto-17β-estradiol) that have been shown to stimulate ERα mediated TGF-β3 gene transcription in bone cells via a novel non-ERE-dependent pathway (54), also interact with the ERβ protein.

Several suspected endocrine disruptors bind weakly to ERα and ERβ protein

The environmental estrogen o, p'-DDT binds weakly to ERα (25, 55) and induces estrogenic effects in female rats. The binding affinity of o, p'-DDT to both ER subtype is 5000- to 10,000-fold lower in comparison to E_2, whereas for the other DDT isomers and metabolites significant radioligand competition was not detected at concentrations up to 10 μM. Apart from DDT, other organochlorine insecticides exhibit estrogenic activity, most notably chlordecone (19, 26). Of these (methoxychlor, chlordecone, and endosulfan) only chlordecone bound to both ER subtypes (Table 1).

Polychlorinated biphenyls (PCBs) are highly toxic halogenated aromatic compounds that are widely distributed in the global ecosystem. Metabolism of PCBs by humans and rodents results in formation of hydroxylated PCBs (OH-PCBs), and several OH-PCBs elicit estrogenic responses in the rat uterus (23). We have investigated the ER binding affinity of a series of OH-PCBs including those identified in human serum (24, 42, 43). In general only minimal, if any competition, was detected (Table 1), except for OH-PCB-K (2', 4', 6'-trichloro-4-biphenylol) and OH-PCB-L (2', 3', 4', 5'-tetrachloro-4-biphenylol), which bound to ERα and ERβ proteins with affinities only 20- to 40-fold lower than E_2. The OH-PCBs K and L have chlorine atom substitutions only in the nonphenolic ring, while all other OH-PCBs tested have chlorine substitutions in both the phenolic and nonphenolic rings. Substitution of one chlorine atom at the para or meta

TABLE 1. RBA of suspected environmental endocrine disruptors for ERα and ERβ from solid-phase (Scintistrip) competition experiments

Compound	RBA[a]	
	ERα	ERβ
17β-estradiol	100	100
17α-estradiol	7	2
Diethylstilbestrol	236	221
2-OH-estrone	2	0.2
17-epiestriol	29	80
16-keto-17β-estradiol	1.3	0.9
Progesterone	<0.01	<0.01
Testosterone	<0.01	<0.01
16α-bromo-17β-estradiol	76	10
5-androstenediol	1	7
4-OH-tamoxifen	257	232
Tamoxifen	4	3
Raloxifene	69	16
o,p'-DDT	0.01	0.02
p,p'-DDT	<0.01	<0.01
o,p'-DDE	<0.01	<0.01
p,p'-DDE	<0.01	<0.01
o,p'-TDE	<0.01	<0.01
p,p'-TDE	<0.01	<0.01
OH-PCB-A	0.1	0.13
OH-PCB-B	0.3	0.2
OH-PCB-C	0.09	0.03
OH-PCB-D	0.3	0.5
OH-PCB-E	0.11	0.11
OH-PCB-F	0.13	0.12
OH-PCB-G	0.06	0.04
OH-PCB-H	0.18	0.23
OH-PCB-K	2.4	4.7
OH-PCB-L	3.4	7.2
OH-PCB-1	0.03	0.02
OH-PCB-2	0.03	0.04
OH-PCB-3	0.09	0.1
OH-PCB-4	0.01	<0.01
OH-PCB-5	0.07	0.06
OH-PCB-6	0.1	0.1
OH-PCB-7	0.1	0.1
4-*tert*-butylphenol	<0.01	<0.01
4-*tert*-amylphenol	<0.01	<0.01
4-*tert*-octylphenol	0.01	0.03
4-octylphenol	0.02	0.07
Nonylphenol	0.05	0.09
Bisphenol A	0.01	0.01
Methoxychlor	<0.01	<0.01
Endosulfan	<0.01	<0.01
Chlordecone	0.06	0.1
4,4'-biphenol	<0.01	0.03

[a] RBA of each competitor was calculated as ratio of concentrations of E_2 or competitor required to reduce the specific radioligand binding by 50% (= ratio of IC_{50} values). RBA value for E_2 was arbitrarily set at 100.

The full names of the OH-PCB and DDT analogs are given in the *Materials and Methods* section.

position in the phenolic ring of OH-PCB-K and OH-PCB-L, respectively, lowers the binding affinity about 20-fold for both ER subtypes (compare OH-PCB-K with OH-PCB-D and OH-PCB-L with OH-PCB-E in Table 1). The very low binding affinity for ERα as well as ERβ protein of the OH-PCBs tested, except for those which have no chlorine atom substitutions in the phenolic ring, is in agreement with previous studies in which radioligand competition experiments were performed using rat or mouse uterus cytosol as a source of ER protein (23, 24, 43).

Alkylphenols are composed of an alkyl group that can vary in size, branching, and position joined to a phenolic ring. Nonylphenol and octylphenol are estrogenic in the breast cancer cell proliferation assay (17, 21, 29), in a recombinant yeast screen with human ERα (27) and in the rat uterus growth bioassay (56), although they are 1000- to 10,000-fold less potent than E_2. Alkylphenols compete with E_2 for binding to both ER subtypes to the same extent; that is nonylphenol > 4-octylphenol > 4-*tert*-octylphenol > 4-*tert*-amylphenol = 4-*tert*-butylphenol (Table 1). The binding affinity increases with the number of C-atoms in the alkylgroup, although it is maximally 1000- to 2000-fold lower for both ER subtypes as compared with E_2. The affinity for ERβ seems to be higher, but more alkylphenols should be tested to see if this is a general finding.

Bisphenol A is the monomer used in the production of polycarbonate plastics, and it shows estrogenic activity in MCF-7 human breast cancer cells as well as in rats (28, 57). Bisphenol A has an affinity 10,000-fold lower than that of E_2 for both ER subtypes (Table 1) and 4,4'-biphenol, which lacks the propane group between the phenolic rings, has a similarly low affinity for ERα and ERβ.

Differential binding of several phytoestrogens to ERα and ERβ protein

The binding affinity of coumestrol to ERβ is 7-fold higher in comparison to ERα, whereas for zearalenone only a very small difference in affinity is detectable (Table 2). Several flavonoids, especially genistein, apigenin and kaempferol have a higher binding affinity (20- to 30-fold more) for ERβ in the solid-phase binding assay (Table 2). The exact position and number of the hydroxyl substituents on the flavone or isoflavone molecule seem to determine the ER binding affinity. For example, the isoflavone genistein has a particular high binding affinity for ERβ, but elimination of one hydroxyl group (daidzein, biochanin A) or two hydroxyl groups (formononetin) causes a great loss in binding affinity. The flavone apigenin has moderate affinity for both ER subtypes and addition of hydroxyl groups (kaempferol, quercetin) does not increase but decreases the binding affinities.

To confirm the sometimes quite large differences in relative binding affinity determined in the solid-phase ligand-binding system (Table 2), which was intended to be an initial screening assay, several compounds were also analyzed in more traditional solubilized receptor ligand binding assays (Fig. 2). This is essentially the same assay as the saturation ligand-binding experiments described in Fig. 1, but now in the competition mode. Again, the binding affinity of 16α-bromo-17β-estradiol was significantly higher (about 4-fold) for ERα, whereas the binding affinity of 5-androstenediol is significantly higher for ERβ, as previously described (15). Furthermore, the relative binding affinity of raloxifene for both ER subtypes is similar in both binding assays. For the phytoestrogens the differences in relative binding affinities (RBA) between the ER subtypes measured in the solid-phase ligand-binding system, are largely confirmed in the solubilized receptor ligand-binding system. Coumestrol binds to ERα with an affinity about 3-fold less than that of E_2 itself, which is in agreement with previously described data (38).

TABLE 2. Binding affinity of various phytoestrogens for ERα and ERβ

Compound	RBA[a] ERα	RBA[a] ERβ	RBA[b] ERα	RBA[b] ERβ
17β-estradiol	100	100	100	100
Coumestrol	20	140	34	100
Zearalenone	7	5	10	18
Isoflavones:				
Genistein	4	87	0.7	13
Daidzein	0.1	0.5	0.2	1
Formononetin	<0.01	<0.01	ND	ND
Biochanin A	<0.01	<0.01	ND	ND
Ipriflavone	<0.01	<0.01	ND	ND
Flavones:				
Apigenin	0.3	6	ND	2
Chrysin	<0.01	<0.01	ND	ND
Flavone	<0.01	<0.01	ND	ND
Flavonols:				
Kaempferol	0.1	3	ND	2
Quercetin	0.01	0.04	ND	ND
Flavanone:				
Naringenin	0.01	0.11	ND	0.2
Chalcone:				
Phloretin	0.2	0.7	ND	ND

RBA of each competitor was calculated as ratio of concentrations of E_2 and competitor required to reduce the specific radioligand binding by 50% (= ratio of IC_{50} values). RBA value for E_2 was arbitrarily set at 100.

[a] RBA determined from solid-phase (Scintistrip) competition experiments.

[b] RBA determined from solubilized receptor competition experiments (Fig. 2).

ND, Not determined.

Coumestrol binds with essentially the same affinity as E_2 to ERβ. The approximately 20-fold difference in binding affinity of genistein observed in the solid-phase assay (incubation at ambient temperature instead of 6 C) is confirmed, although the relative binding affinity compared with E_2 is, especially for ERβ, lower (RBA = 87 in Table 2 *vs.* RBA = 13 in Fig. 2). Receptor-binding affinity is a function of temperature and equilibrium time, and for steroid receptors the time necessary for equilibration of receptor-radioligand complexes in the presence of competitor may be up to 1000 min at the lower temperature (58). Because both ligand-binding systems used incubation times of 18–20 h, it is unlikely that this apparent discrepancy is caused by lack of equilibration. For naringenin, apigenin and kaempferol complete displacement of radioligand from the ERα protein could not be obtained (Fig. 2), and the competition curves are nonparallel for the ER subtypes. This could point to binding-site heterogeneity, but further investigations are needed to clarify this point.

Suspected endocrine disruptors stimulate the transcriptional activity of ERα and ERβ

In radioligand competition assays only compounds able to displace or compete with the radioligand for binding to the receptor are detected. Furthermore, ligand-binding assays do not disclose the biological activity of a compound, *i.e* whether it is an agonist or an antagonist. Animals have traditionally been used for the biological profiling of compounds; however, these assays are costly and time-consuming. An alternative for initial characterization of compounds is a cell based transcription assay system, using a reporter gene under the transcriptional control of a specific receptor.

Human embryonal kidney 293 cells were transiently cotransfected with a luciferase enzyme reporter gene construct containing three copies of a consensus ERE in front of a TATA-box, together with human ERα or human ERβ expression plasmids. As shown in Fig. 3, E_2-stimulated reporter gene activity by ERβ was lower when compared with activity obtained by ERα. Also, half-maximal activation (EC_{50}) is reached at a lower concentration of E_2 for ERα than for ERβ (about 5 pM and about 50 pM, respectively). The fold induction was relatively high, and therefore this transactivation assay using embryonal kidney cells was considered to be very suitable to estimate the estrogenic activity of compounds with low binding affinity.

To obtain an impression of the transcription stimulating activity of the compounds tested in the radioligand-competition assay, a selection of these compounds was tested at concentrations up to 1000 nM in the transactivation assay. It should be noted that in Table 3 the (maximal) transcriptional activity at a relatively high concentration of 1000 nM is shown, while in Fig. 4 the transcriptional activity is shown at various concentrations as percentage of the maximal induction by E_2 for each ER subtype separately. The measured relative transactivation activities of the suspected endocrine disruptors (Table 3 and Fig. 4) are comparable with results from the radioligand competition assays, which showed affinities up to 10,000-fold lower than E_2. The OH-PCB-D and OH-PCB-E compounds, which have a very low binding affinity for both ER subtypes (Table 1) do not display any agonist activity. Also, no antagonist activity could be detected in experiments with various E_2 concentrations and up to a 1000-fold excess of OH-PCB-D or OH-PCB-E (not shown). On the other hand, the OH-PCB-K and OH-PCB-L compounds, which have a higher binding affinity (Table 1), are relatively strong agonists for both ER subtypes. With regard to the organochlorine insecticides, the lack of significant binding affinity of methoxychlor, endosulfan and p, p′-DDT is consistent with their low agonist activities. Chlordecone (Kepone) is a weak agonist for ERα, but it has no agonist activity on ERβ despite the fact that the binding affinities are similar (Table 1). Neither on ERβ nor on ERα any antagonist activity of chlordecone could be detected in experiments in which up to a 10,000-fold excess of chlordecone was incubated together with E_2 (not shown). Bisphenol A is an equally strong agonist for ERα as for ERβ, and the same is true for 4,4′-biphenol, which differs from bisphenol A in that it lacks the propane group between the phenolic rings. No agonist activity of the antiestrogens tamoxifen and ICI-182780 could be detected on ERβ, whereas tamoxifen had some agonistic activity on ERα (Table 3). Transcriptional stimulation observed for suspected endocrine disruptors was dependent on cotransfected ERα or ERβ, confirming that the transcriptional activation was mediated by the estrogen receptor (not shown).

Flavonoids, coumestrol, and zearalenone stimulate transcriptional activity mediated by ERα and ERβ

Transactivation activity of phytoestrogens (Table 3 and Fig. 4) was measured after incubation of transfected cell cultures with concentrations of up to 1000 nM. In humans,

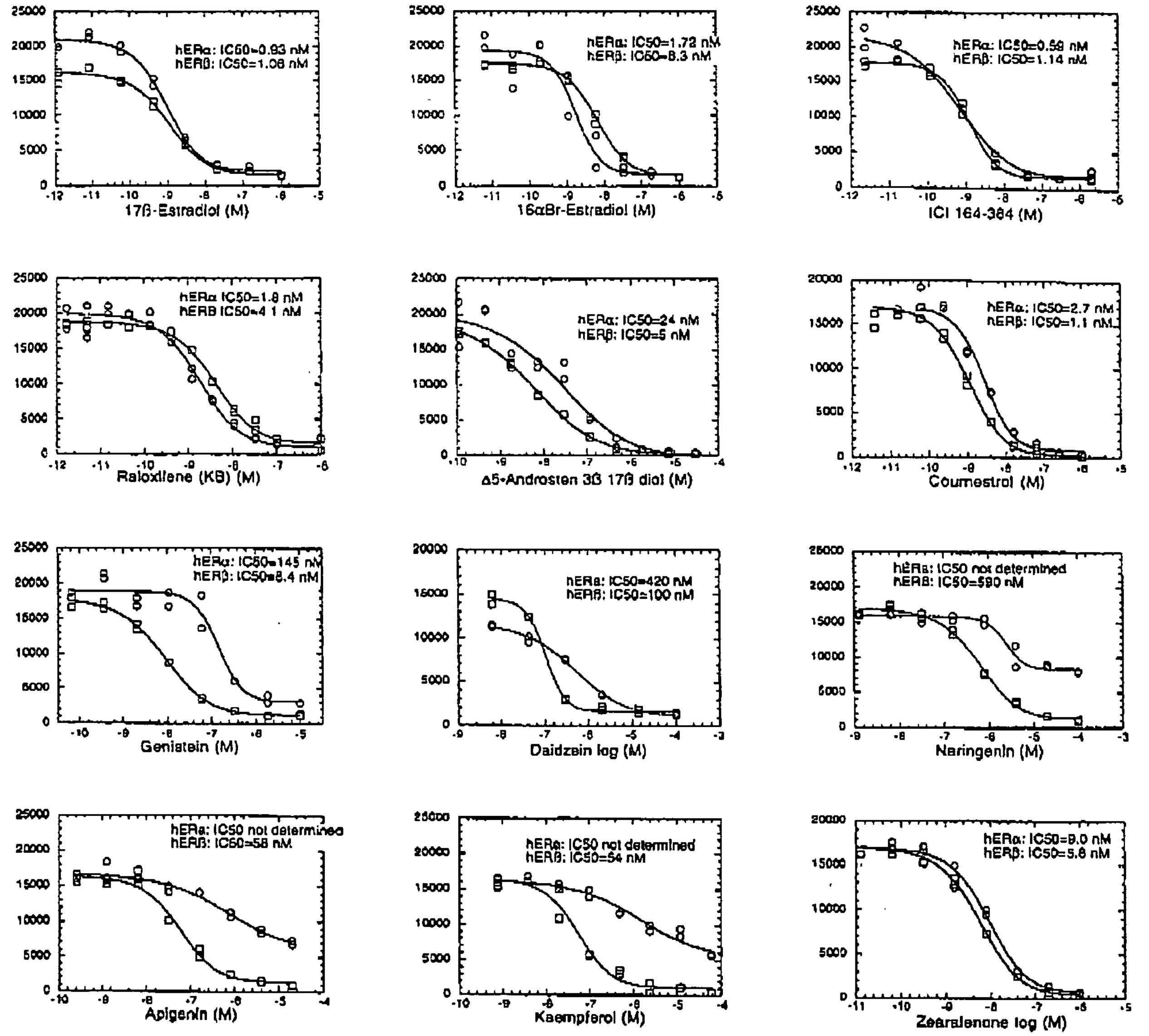

FIG. 2. Competition (solubilized receptor assay) by several nonradioactive (phyto)-estrogens and antiestrogens for ^{3}H-17β-estradiol binding to ERα (○) and ERβ protein (□). Incubation was for 18 h at 6 C, and bound and unbound radioligand were separated as described for the solubilized receptor assay. Abscissa, log M of compound; ordinate, dpm bound radioligand.

peak serum concentrations of total daidzein and total genistein of 500-1000 nM can be reached after consumption of meals rich in soybeans or soybean protein extracts (41, 59). The phytoestrogens with binding affinities 10,000-fold or more less than E_2 (formononetin, ipriflavone, chrysin, quercetin) have very low or no agonistic activity. Also, the binding affinity of biochanin A is more than 10,000-fold less than E_2 for both ER subtypes (Table 2); however, it has relatively strong agonistic activity (Table 3). Biochanin A is the 4'-methylether of genistein, and it has been shown that MCF-7 breast tumors cells can convert biochanin A to genistein (60). A similar partial conversion of biochanin A to genistein by the 293 embryonal kidney cell line used for the transactivation assay might explain the observed discrepancy. The estrogenic potency of the remaining flavonoids (daidzein, apigenin, kaempferol, naringenin, phloretin) at a concentration of 1000 nM is in line with the observed 100- to 500-fold lower binding affinities for both ER subtypes. Based upon these data (Fig. 4) and additional dose-response curves not shown, a ranking of the estrogenic potencies of the phytoestrogens is as follows: 17β-estradiol ≫ zearalenone = coumestrol > genistein > daidzein > apigenin = phloretin > (biochanin

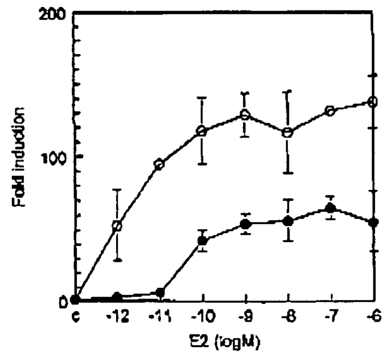

FIG. 3. Activation of transcription by E_2 in human embryonal kidney 293 cells. Cells were transfected with ERE-TATA-Luc reporter plasmid, and pSG5-hERα (○) or pSG5-hERβ (●) expression plasmid. After 16 h, the medium was changed and E_2 or vehicle was added (c = control). After 24 h incubation, the cells were lysed and the reporter gene activity was measured. Results are expressed as fold induction ±SD from two different experiments with each concentration in triplicate.

A) = kaempferol = naringenin > formononetin = ipriflavone = quercetin = chrysin for ERα and 17β-estradiol ≫ genistein = coumestrol > zearalenone > daidzein > (biochanin A) = apigenin = kaempferol = naringenin > phloretin = quercetin = ipriflavone = formononetin = chrysin for ERβ. Although these phytoestrogens are clearly less potent at inducing a biological response than E_2, some of them (genistein, zearalenone, coumestrol) are able to generate a response of the same or almost the same magnitude as that produced by the physiological hormone at concentrations of 10–100 nM. In fact, at high concentrations (1000 nM) the estrogenic potency of genistein was greater than that of E_2.

For zearalenone, antagonistic activity could be detected during incubation of ERβ transfected cell cultures with 1 nM E_2 and 100- to 1000-fold excess zearalenone. No antagonistic activity of zearalenone could be detected when cell cultures were transfected with ERα (Fig. 5). In fact, zearalenone is a full agonist for ERα and a mixed agonist-antagonist for ERβ in this transactivation assay system (Fig. 5). For genistein (Fig. 5) and the other phytoestrogens, no antagonism could be detected. Genistein and coumestrol are full agonists on ERα as well as ERβ, although weaker than E_2 (Fig. 5). The half maximal activity for genistein (Fig. 4) on ERα is reached at about 20 nM (compared with about 0.005 nM for E_2) and for ERβ at about 6 nM (compared with about 0.05 nM for E_2). Therefore, although the 20-fold higher binding affinity of genistein for ERβ (Table 2) is reflected in only a 3-fold lower EC_{50} value, the relative estrogenic potency of genistein on ERβ is about 30-fold higher compared with the potency on ERα (estrogenic potency $0.005/20 \times 100 = 0.025$ for ERα and $0.05/6 \times 100 = 0.8$ for ERβ with $E_2 = 100$). Similar calculations for coumestrol (Fig. 4) reveal an estrogenic potency of 0.05 for ERα and 0.5 for ERβ with $E_2 = 100$. So, the higher binding affinity of coumestrol and genistein for ERβ is reflected in a clearly higher estrogenic potency. The transcriptional activity of the phytoestrogens was dependent on cotransfected ERα or ERβ expression plasmids, confirming that the transcriptional activity was mediated by the estrogen receptor protein (not shown).

TABLE 3. Relative transactivation activity[a] of various compounds for ERα and ERβ

Compound	ERα	ERβ
17β-estradiol	100	100
Diethylstilbestrol	117	69
Tamoxifen	6	2
ICI-182780	1	2
o,p'-DDT	54	10
p,p'-DDT	7	2
OH-PCB-D	3	3
OH-PCB-E	1	1
OH-PCB-K	77	62
OH-PCB-L	68	41
4-*tert*-octylphenol	70	51
4-octylphenol	61	57
Nonylphenol	62	34
Bisphenol A	50	41
Methoxychlor	9	2
Endosulfan	6	1
Chlordecone	27	1
4,4'-biphenol	59	72
Coumestrol	102	98
Zearalenone	91	27
Genistein	198	182
Daidzein	97	80
Formononetin	6	2
Biochanin A	36	63
Ipriflavone	11	3
Apigenin	60	49
Chrysin	1	2
Flavone	2	2
Kaempferol	35	59
Quercetin	3	2
Naringenin	36	45
Phloretin	49	10

[a] The relative transactivation activity of each compound was calculated as the ratio of luciferase reporter gene induction values of each compound at a concentration of 1000 nM and the luciferase reporter gene induction value of 17β-estradiol at 1000 nM. The *trans*-activation activity of 17β-estradiol was arbitrarily set at 100.

Discussion

The ER binds a large number of compounds that exhibit remarkably diverse structural features. In fact, the estrogen receptor is probably unique among the steroid receptors in its ability to interact with a wide variety of compounds. This is true for the ERα subtype but also for the ERβ subtype. Binding studies have provided a description of the ligand structure-estrogen receptor binding affinity relationships and a model for the ligand binding site (61). This model indicated that the whole E_2 skeleton, that is, the aromatic A-ring, the B- and C-rings, and the OH-group in the D-ring contribute significantly to receptor binding. It was also predicted that the receptor-bound ligand is completely surrounded by the receptor with minimal exposure to solvent. The recently determined crystal structure of the ERα ligand-binding domain complexed with E_2 provided important confirmation for this model (62). The phenolic hydroxyl group of the A-ring of E_2 nestles between two α-helices and makes several direct hydrogen bonds. This pincer-like arrangement around the A-ring imposes an absolute requirement on ligands to contain an aromatic ring, whereas the remainder of the binding pocket can accept a number of different hydrophobic groups. The overall promiscuity of the ER can be attributed to the size of the binding cavity, which has a

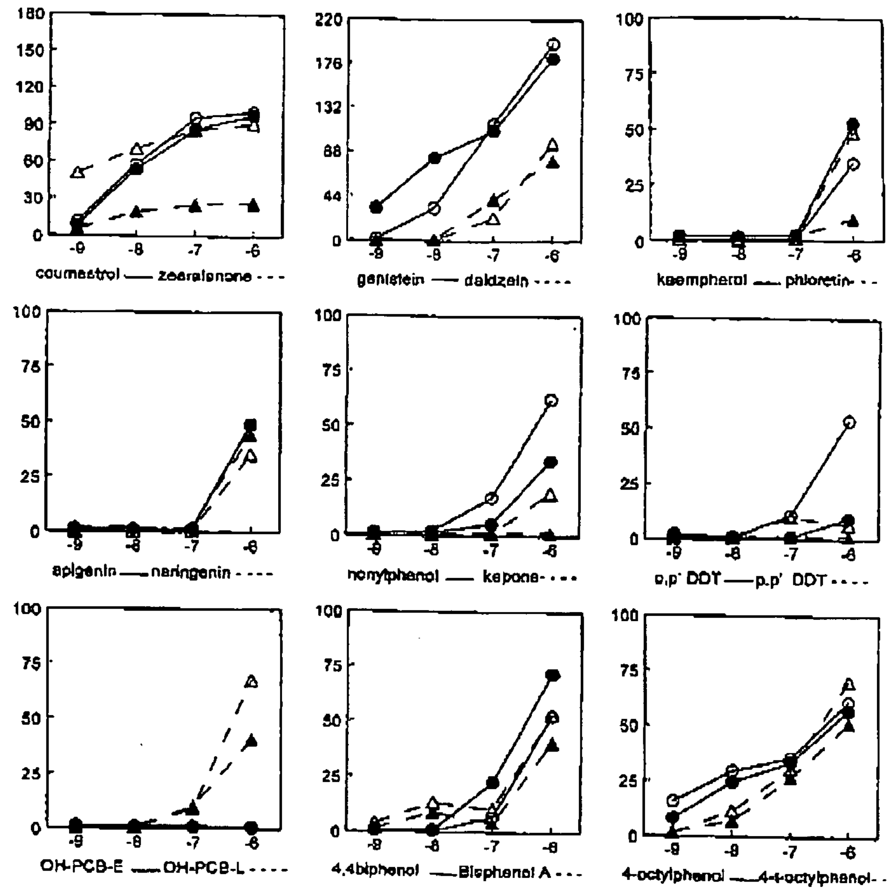

FIG. 4. Activation of transcription by various estrogenic chemicals and phytoestrogens. The experiment (two different experiments with each point in triplicate) were done as described in *Materials and Methods*. ERα = ○ or △ and ERβ = ● or ▲. Abscissa, log M of compound; ordinate, transcriptional activity as percentage of the maximal induction by E_2 for each ER subtype.

volume almost twice that of the E_2 molecular volume. The length and the width of the E_2 skeleton is very well matched by the receptor, but there are large unoccupied cavities opposite the B-ring and the C-ring of E_2 (62). Obviously, several phytoestrogens (coumestrol, genistein) fit very well into the available space, certainly for the ERβ protein. It is difficult to understand why other phytoestrogens do not exhibit higher binding affinities because the orientation of the nonsteroidal ligands within the binding pocket is unknown.

Although most of the estrogenic chemicals examined in this study contain at least one aromatic ring with a hydroxyl group, their relative affinities are generally 1000- to 10,000-fold lower than E_2. The complexes formed with the ER are probably very unstable, as shown for various alkylphenols (63), and it is likely that these compounds do not completely enter the ligand-binding pocket. The observed radioligand competition might reflect blockade of E_2 entrance to the binding site or interaction with another low affinity site that causes a change in the high affinity E_2 binding site. If this is true, it will be difficult to use quantitative-structure activity relationship (QSAR) models developed using ligands that bind with high affinity to predict those chemical structures from compound libraries that might disrupt development and reproduction in wildlife, as has been proposed recently (64). Despite their very low binding affinities, several of the suspected endocrine disruptors exhibit estrogenic activities in the transactivation assay system with ERα as well as ERβ, albeit only at a potency that is more than 1000-fold lower than that of E_2. Obviously, these compounds can induce at least partially the conformational changes involved in the formation of a transcriptionally competent activation function in the ligand-binding domain (62). No striking differences in the relative binding affinities for the tested compounds between ERα and ERβ could be detected. Both ER subtypes could therefore be involved in the described developmental and reproductive effects of estrogenic chemicals, depending on their fetal tissue distribution pattern (17–22, 30–35).

The relatively low estrogenic potencies of suspected endocrine disruptors suggests that these chemicals alone are unlikely to produce adverse effects during fetal development (21). These compounds occur as mixtures in the environment and diet, and synergistic transcriptional activation of binary mixtures of weakly estrogenic chemicals have been described (65). However, in subsequent detailed studies these synergistic interactions for ER ligand-binding or transactivation could not be confirmed (65, 66). Some suspected endocrine disruptors have been shown to interact not only with the ER but also with the androgen receptor or to interfere

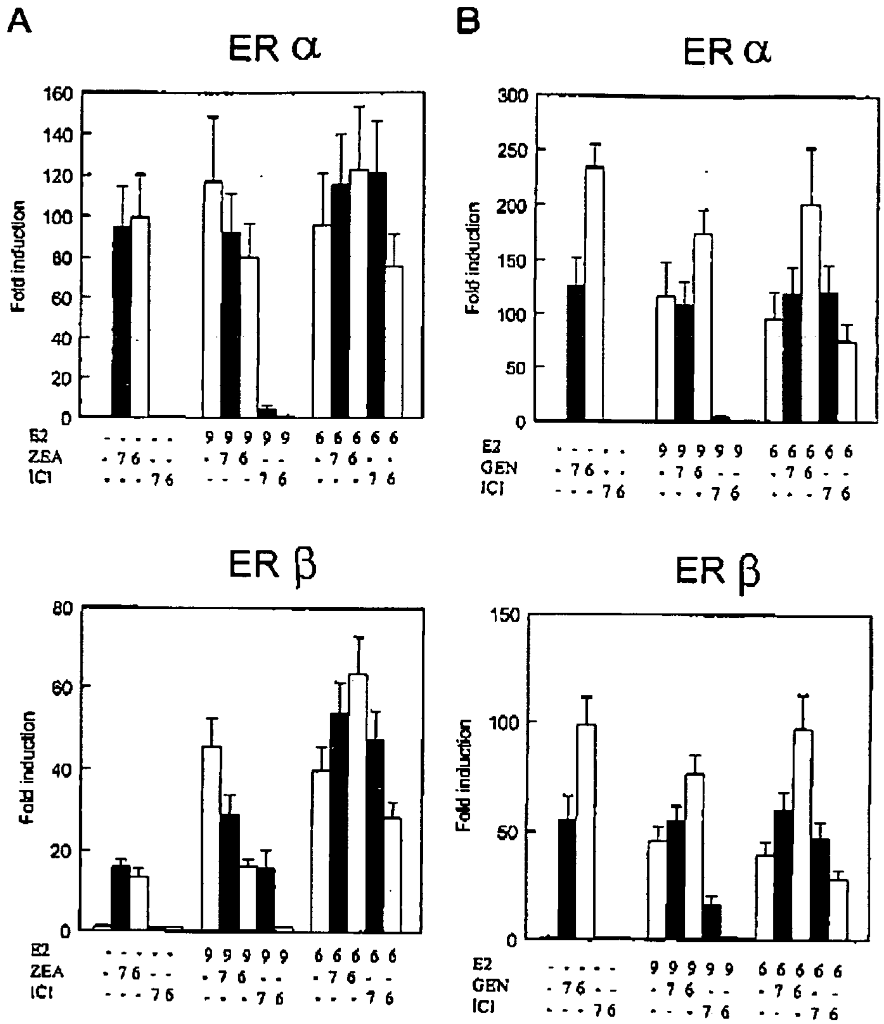

FIG. 5. Activation of transcription by zearalenone and genistein in the absence or presence of E_2. A, Transfected cell-cultures were incubated (conc. shown as -log M) with zearalenone (ZEA), ICI-182780 (ICI) or E_2 alone or in combinations as indicated. Results are expressed as fold induction over vehicle only incubation ±SD for two different experiments with each combination in triplicate. B, Transfected cell-cultures were incubated (concentration shown as -log M) with genistein (GEN), ICI-182780 (ICI) or E_2 alone and in combinations as indicated. Results are expressed as fold induction over vehicle only incubation ±SD for two different experiments with each combination in triplicate.

with steroid hormone synthesis or metabolism (20). Combined effects of mixtures of endocrine disruptors with a different mode of action could in this way result in synergistic responses *in vivo* (20 and references therein). Most suspected endocrine disruptors have been tested in *in vitro* systems (radioligand competition, transactivation assays) and these tests may underestimate or overestimate their *in vivo* estrogenic potency. The estrogenic potency of bisphenol A *in vitro* is 1000- to 5000-fold lower than that of E_2, but *in vivo* bisphenol A was rather effective in stimulating PRL release from the pituitary (57). Development of *in vivo* reporter systems for the assessment of the estrogenic activity of suspected endocrine disruptors might be necessary. If the ligand-binding domain of the ER is fused to a DNA-recombinase, the recombinase activity is controlled efficiently by either agonistic or antagonistic ligands (67, 68). Transgenic mice could be produced in which activation of the recombinase hybrid is detected via elimination of a disruption in a reporter gene (for instance galactosidase or lac Z), thus enabling the use of a simple histochemical reaction in mouse embryos to study the activity of suspected estrogenic chemicals. Of all the suspected endocrine disruptors tested the OH-PCB-K and OH-PCB-L compounds have the highest binding affinity (Table 1), but this is not reflected in the transcription activation potency because compounds with lower binding affinity have equally high estrogenic activity (Table 1 and Table 3 and dose-response curves not shown). The estrogenic potency of compounds is a complicated phe-

nomenon that is the result of a number of factors, such as differential effects on the transactivation functionalities of the receptor, the particular coactivators recruited and the cell- and target gene promoter-context (62). The apparently lower transcriptional activity of ERβ compared with ERα (Fig. 3) has also been reported in transient transfection experiments using different cell lines (CHO, COS, HeLa) and reporter gene constructs (11–13, 69). In contrast, in human osteosarcoma or human endometrial carcinoma cells the transcriptional activity of ERβ was higher than that of ERα (70). The reason for these differences in transcriptional activity of the ER subtypes is at the moment unknown, but it might reflect differential expression of transcriptional coactivators or differential stability of the receptor proteins.

Several phytoestrogens have a higher binding affinity for the ERβ protein (Fig. 2), and both ER subtype transcripts are present in prostate and breast tumor biopsies, although expression levels vary widely (14, 71). In several epidemiological studies, an inverse relation has been suggested between the risk of prostate cancer or breast cancer and the intake of soy foods or the urinary excretion of phytochemicals (39–41, 72–74), although in other studies this could not be confirmed (72). The possibility still exists that the association between reduced breast- and prostate cancer risk and phytoestrogen intake is not causal, and merely results from some other dietary characteristic. Despite the inconclusive epidemiological findings, several putative mechanisms that could account for the hypothesized chemopreventive effects of phytoestrogens have been proposed. Most prominently, phytoestrogens have been suggested to exert strong antiestrogenic effects, thereby inhibiting development of hormone-related cancers (39, 72). In our study, only zearalenone exhibited some antagonistic activity. All other phytoestrogens, including the flavonoids that are present in soy foods, showed only agonistic activity. In previous *in vitro* studies, involving ERα, only agonistic or at best partial antagonistic activities instead of complete antagonistic activities were reported (36–38, 75). Several other mechanisms for the proposed chemopreventive effects of flavonoids have been suggested, including induction of cancer cell differentiation, inhibition of protein tyrosine kinases, suppression of angiogenesis, and direct antioxidant effects (41, 76). These alternative mechanisms generally occur at flavonoid concentrations much higher (>5 μM) than the concentrations at which estrogenic effects are detected (<100 nM), and show a different structure-activity relationship; moreover, the effects are observed in cells in the absence of ER expression, and therefore it seems unlikely that all of these effects are ER mediated (41, 77, 78). On the other hand, because both ER subtypes are expressed in bone and the cardiovascular system (4, 79–81) and given the quite strong estrogenic activity of certain phytoestrogens, the potential beneficial effects of increased food intake of phytoestrogens in the prevention of postmenopausal osteoporosis and cardiovascular diseases should be further investigated (82).

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